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Association between clinical presentation, biogroups and virulence attributes of *Yersinia enterocolitica* strains in human diarrhoeal disease

A. P. BURNENS*, A. FREY AND J. NICOLET

Swiss National Reference Laboratory for Foodborne Diseases, Institute for Veterinary Bacteriology, University of Berne, Länggass-Strasse 122, 3012 Berne, Switzerland

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SUMMARY

Traditionally the enteric pathogen *Yersinia enterocolitica* has been differentiated into biogroups. Despite being considered as non-pathogenic, biogroup 1A isolates have constituted a sizeable fraction of strains from patients with gastroenteritis in many reports. To establish a potential clinical significance for biogroup 1A isolates of *Y. enterocolitica*, clinical disease in patients with gastroenteritis excreting such isolates was compared with symptoms among patients found infected with pathogenic biogroups. Clinical data and isolates of 66 patients from whom *Y. enterocolitica* had been isolated by direct plating were available for study. There was an association between patient age below 3 years and infection with 'pathogenic' *Y. enterocolitica*. The severity of gastroenteritis and other symptoms, however, did not depend on the biogroup, or the presence of the virulence plasmid in the yersinia strain isolated from the patients. Strains belonging to biogroup 1A of *Y. enterocolitica* showed two clusters of ribotypes, one of which encompassed most isolates recovered from humans, the other being associated with environmental isolates. This might indicate the existence of human-adapted and potentially pathogenic strains among biogroup 1A of *Y. enterocolitica*.

INTRODUCTION

Yersinia enterocolitica has been described as an agent of bacterial gastroenteritis worldwide (reviewed in [1]). Its prevalence varies widely between geographic regions, and in colder climates *Y. enterocolitica* may rival salmonellae as cause of infectious gastroenteritis [2]. Strains of *Y. enterocolitica* are traditionally differentiated into biogroups, of which biogroups 1B, 2, 3, 4 and 5 have been designated as pathogenic, whereas the biogroup 1A is considered to be non-pathogenic [3]. Strains of the former biogroups 3A and 3B have recently been reassigned to new species of the genus, i.e. *Y. mollaretii* and *Y. bercovieri* [4]. Among the pathogenic strains of *Y. enterocolitica*, biogroups 2 and 4 are frequently recovered from humans in Europe, whereas biogroup 1B is most prevalent in the USA. The remaining pathogenic biogroups are considered primarily animal pathogens, although strains of biogroup 3 are found occasionally in humans [5]. The pathogenic mechanisms involved in infections by yersiniae have been widely studied at a molecular level. Strains of *Y. enterocolitica* belonging to pathogenic biogroups have been shown to possess an array of virulence-associated genetic elements. In particular, all pathogenic strains have a 44–47 Md virulence plasmid [3], as well as chromosomal virulence genes, such as the enterotoxin gene *yst* [6] and the invasion-associated gene *ail* [7]. There is no doubt about the clinical significance of infections with pathogenic *Y. enterocolitica* and therefore a simple biogrouping scheme is considered part of the standard laboratory workup for this organism in reference textbooks [8]. However, the role of non-pathogenic strains in human disease has been more controversial [9].

In Switzerland, the annual incidence of human *Y. enterocolitica* infections is estimated to be 1·4 per 100000, based on notifications to the Federal Office of Public Health, and compared to 82 per 100000 for *Salmonella* spp. [10]. Among the human isolates of *Y. enterocolitica* sent to the Swiss National Reference Laboratory for confirmation and typing, about 60% of the strains had been of the non-pathogenic biogroup 1A in previous years. In order to study the clinical significance of *Y. enterocolitica* belonging to biogroup 1A and to obtain an unbiased estimate of the occurrence of such isolates, all strains isolated in two clinical microbiology laboratories from humans during the study period were analysed. Because we hypothesized that clinical symptoms might differ between patients infected with pathogenic and non-pathogenic yersiniae, medical histories of these patient groups were compared. A special effort was made to obtain information on potential late sequelae of yersinia infection. Some patients with disease due to *Y. enterocolitica* develop symptoms of arthritis and erythema nodosum as late complications [1]. The possibility of an association between pathogenicity of the infecting strain and the development of these complications had not been addressed before.

METHODS

Bacterial strains, culture conditions and laboratory procedures

During the study period, January 1992 to June 1994, all isolates identified as *Yersinia enterocolitica* in two clinical microbiology laboratories were analysed. The laboratory of Dr O. Lutz, Winterthur, Switzerland contributed 16 strains, and the laboratory of Dr Viollier, Basel, Switzerland another 19 strains. An additional 36 sporadic isolates of yersinia referred on a voluntary basis to the Swiss National Reference Laboratory for Foodborne Diseases for confirmation and typing by another 16 clinical microbiology laboratories were also analysed. All laboratories used commercially available formulations of the CIN *Yersinia* selective agar [11] for culture of yersinae, at temperatures between 22 °C and 30 °C for 48 h, depending on the standard procedures of the laboratories. No enrichment cultures for yersiniae were performed. Other enteric pathogens were isolated by each laboratory with their usual procedures, and the presence of other pathogens besides yersinia was recorded. Four reference strains from the collection of H. H. Mollaret, Institut Pasteur, Paris, were used for comparison: IP 134, biogroup 4, serogroup O3, IP 383, biogroup 2, serogroup O9, IP 1105, biogroup 1B, serogroup O8, and IP 885, the reference strain for serogroup O5, 27.

For culturing, strains were never incubated at temperatures above 30 °C to minimize loss of the virulence plasmid. The strains of yersinia were identified to species and biogroup level in standard media [12], using the following biochemical reactions at 30 °C: decarboxylation of lysine and ornithine, production of urease, deamination of phenylalanine, hydrolysis of Tween 80 [13], pyrazinamide [14], ONPG, and aesculin, fermentation of glucose, mannitol, lactose, saccharose, rhamnose, dulcitol, raffinose, cellobiose, melibiose, inositol, sorbitol, xylose, and mucate, utilisation of malonate, citrate and acetate, production of gas, acetoin, indole, and H_2S, and the methyl-red reaction. Motility was tested at 30 °C and 37 °C, and congo-red binding as well as low-calcium-response were determined on congo-red magnesium oxalate agar [15]. Slide agglutinations with rabbit sera against serogroups O3, O9, O5, and O8 of *Y. enterocolitica* were performed with commercially available reagents (catalogue no. 311103 to 311106, Denka Seiken, Japan).

Patient characteristics and clinical data

Stool cultures for yersinia had been performed in the laboratories at the request of the patients' physicians. All medical histories were reviewed with the physician by telephone interview by one investigator (A. F.), and the data were coded in Epi Info for analysis [16]. Information was sought on clinical signs including diarrhoea, abdominal cramps, symptoms of appendicitis (right lower quadrant pain, leucocytosis, and guarding), gross blood in stools, fever, septicaemia, requirement for antibiotic treatment, duration of antimicrobial treatment, duration of symptoms, and hospitalization. The medical history was also checked for illnesses predisposing to yersiniosis or late complications [1], especially diabetes, haemochromatosis, liver cirrhosis, iron overload, and HLA B27 histocompatibility antigen, or general medical conditions associated with decreased host defences, like cancer and HIV infection. A follow-up call at least 3 weeks after the acute episode was made to ascertain the presence of late complications, i.e. arthritis and erythema nodosum [1]. Food histories were not

recorded but foreign travel was recorded as a potential factor influencing the risk of infection with *Y. enterocolitica* biogroups.

Molecular biology techniques

Genomic DNA was extracted by a modified guanidium thiocyanate microprocedure [17]. Nucleases were inactivated by extraction with a mixture of phenol-chloroform-isoamyl alcohol (25:24:1) instead of chloroform alone [18]. For the colony-blot, representative colonies of each strain of *Y. enterocolitica* were inoculated onto filters marked with a grid (Millipore HAWG 047 S3, Millipore Corporation Bedford, MA 01730) overlying Luria-Bertani agar plates [18], and incubated at 30 °C overnight. Colonies were lysed and DNA fixed to the filters essentially as already described [19], by placing the filters on filter paper (Whatman 3 MM) soaked with 0·5 M-NaOH and 1·5 M-NaCl for 30 min, then 1 M-Tris-HCl pH 7·5, 1·5 M-NaCl for 10 min, afterwards with buffer A (50 mM-Tris-HCl pH 8·0, 125 mM-NaCl, 10 mM-EDTA, and 0·5% SDS) for 10 min, finally with buffer A containing 5 mg/ml lysozyme for 10 min. After 2 h of fixation at 80 °C in a vacuum oven and 1 h of treatment with buffer A containing 0·2 mg/ml proteinase K at 50 °C, the filters were washed in a buffer containing 50 mM-Tris-HCl pH 7·6 and 1 M-NaCl, 1 mM-EDTA, and 0·1% SDS for 1 h at 50 °C.

Non-radioactively labelled probes for hybridization were prepared as follows. For the *ail*-probe, plasmid pVM103 [20] was cut with the restriction enzymes *Cla*I and *Ava*I according to the manufacturers instruction (Boehringer Mannheim, Rotkreuz, Switzerland), and the fragments were separated by agarose gel electrophoresis. The 1·3 kb *Cla*I-*Ava*I fragment was extracted from the gel [18], and labelled with DIG-dUTP by random priming following the instructions of the manufacturer (Boehringer). Digoxigenin labelled probes targeting the *Y. enterocolitica yst*-gene and 16S ribosomal RNA sequences were prepared by PCR. To a standard 100 μl PCR mixture, containing 2·5 U of Taq DNA polymerase (Boehringer), 170 μM of dNTP, and 0·25 μM of oligonucleotide primers in 10 mM-Tris pH 8·3, 1·5 mM-$MgCl_2$, 50 mM-KCl, and 0·005% each of Tween 20 and NP-40, DIG-dUTP (cat no. 1093088, Boehringer) was added in a concentration of 50 μM. For the *yst*-probe, genomic DNA of a reference isolate of *Y. enterocolitica* O3 biogroup 4 (638–93) was used as template, purified pKK3535 plasmid DNA, containing the complete *E. coli rrnB* operon [21] served as template for the *rrn*-probes. The sequences of the oligonucleotide primers were 5′-AAT GCT GTC TTC ATT TGG-3′ and 5′-CGG GAT TGC AAC ATA CAT-3′ for the *yst*-probe [6], and 5′-ATT GAA GAG TTT GAT CAT GGC TCA-3′ and 5′-CTC CCA TGG TGT GAC GGG CGG TGT GTA-3′ for the 16S and 5′-CTT AGA AGC AGC CAT CAT TT-3′ and 5′-CTT TTA TCC GTT GAG CGA TG-3′ for the 23S *rrn*-probes, respectively. Amplification was performed in a DNA thermal cycler (Perkin-Elmer Cetus) and was taken through 35 cycles with 1 min each at the following temperatures: denaturation at 94 °C, annealing at 50 °C for the *yst*-probe, 48 °C for the 16S *rrn*-probe, and 56 °C for the 23S *rrn*-probe, and elongation at 74 °C.

For ribotyping, the DNA was cut with the restriction enzymes *Pvu*II which had been shown to be most discriminatory on preliminary analysis, according to the instructions of the manufacturer (Boehringer). DNA was transferred to nylon membranes (Boehringer) by alkaline transfer with 0·4 M-NaOH in a 2016 VacuGene vacuum blotting device (LKB, Bromma, Sweden). Hybridization and detection were performed both for colony blots as well as Southern blots following the instructions of the manufacturer (DNA labelling and detection kit non-radioactive, Boehringer). The stringency wash after hybridization was carried out twice in 0·2 × SSC (30 mM-NaCl, 3 mM-trisodium citrate pH 7·0) with 0·1% SDS for 15 min at 68 °C.

RESULTS

During the study period, 81 isolates of presumptive *Yersinia enterocolitica* were analysed. Among these, 10 strains belonged to other, recently described species of *Yersinia*; five were identified as *Y. bercovieri*, two each as *Y. kristensenii* and *Y. intermedia*, and one was *Y. frederiksenii*. Of the 71 isolates identified as *Y. enterocolitica*, 26 belonged to the non-pathogenic biogroup 1A, 22 to biogroup 2, 20 to biogroup 4, and 3 to biogroup 3. Table 1 summarizes the ability of different methods to discriminate reliably between pathogenic biogroups of *Y. enterocolitica* and the non-pathogenic biogroup 1A. Several biochemical tests were useful for discrimination and gave comparable results. Prototrophy was also found a very useful discriminating character. *Yersinia enterocolitica* belonging to pathogenic biogroups uniformly failed to utilize sodium acetate in a mineral base medium,

Table 1. *Sensitivity and specificity of various laboratory tests to detect pathogenic* Y. enterocolitica *compared to full biogrouping**

Test	Sensitivity (%)	Specificity (%)
Pyrazinamidase	100	100
Aesculin hydrolysis on bile aesculin agar	100	100
Acetate utilization	100	96
Agglutination†	96	73
Hybridization with *ail* probe	100	96
Hybridization with *yst* probe	100	100
Tiny red colonies on CR-Mox agar	100	100

* There were 45 isolates of pathogenic biogroups, and 26 isolates of nonpathogenic biogroup 1A.

† Agglutination in O3, O9, O5, and O8 antisera.

whereas all but one isolate of biogroup 1A were positive in this test. Slide agglutinations with rabbit sera against the predominant pathogenic serotypes were less satisfactory. However, elimination of the results of agglutination in the O5 antiserum increased the specificity of this test to 100%, with only 2% loss in sensitivity.

Except for one strain of biogroup 1A reacting with the *ail*-probe, genetic methods and full biogrouping gave identical results. In addition, all pathogenic isolates were carrying the virulence plasmid, as measured by calcium dependence and congo-red binding (Table 1). This was important for our study, because it was ascertained that the patient isolates either contained the full set of virulence attributes defined so far, or lacked them completely, rendering the comparison more meaningful.

The clinical signs and symptoms of patients infected with *Y. enterocolitica* of biogroup 1A were compared with those of patients in whom *Y. enterocolitica* of pathogenic biogroups had been isolated. Data for two patients infected with biogroup 1A isolates, an 89-year-old female and an 88-year-old male, were not available because either the patient file or the doctor could not be located. Four isolates belonging to the pathogenic biogroup 2 were isolated in three children and their mother during a family outbreak, probably due to faecal contamination of the drinking water source in their private well by runoff from a manured meadow. Only the index case, a 4-year-old girl, was included in the analysis. Thus, with the other 3 family cases excluded, and data missing for 2 cases, there were 66 of the original 71 patients available for study.

Table 2. *Clinical signs and findings in 66 patients infected with pathogenic or non-pathogenic isolates of* Y. enterocolitica

Finding	Percent prevalence among patients infected with *Y. enterocolitica* belonging to	
	Non-pathogenic biogroup 1A (n = 23*)	Pathogenic biogroups (n = 43*)
Diarrhoea	91	88
Abdominal cramps	23	33
Vomiting	9	16
Blood in stool	0	9
Fever above 38 °C	27	29
Pseudoappendicitis	9	9
Treatment with antibiotic	48	57
Hospitalization	14	10
Septicaemia	0	2
Duration > 2 weeks	33	45
Age below 3 years	4	33‖
Predisposing medical conditions†	9	7
Other enteric pathogens found‡	13	5
Foreign travel	24	17
Arthritis§	0	0
Erythema nodosum§	0	0

* Number of patients in group.

† Antineoplastic drugs (2), aortic aneurysm (1), diabetes mellitus (1), IgG2 deficiency (1).

‡ Salmonella (2), giardia (2), rotavirus (1).

§ Late complications ascertained by follow-up telephone call.

‖ Chi square 6·79, $P < 0{\cdot}01$.

The results are presented in Table 2. No significant differences were found, except for the fact that pathogenic *Y. enterocolitica* predominated in children below the age of 3 years. The mean age for the patients infected with biogroup 1A isolates of *Y. enterocolitica* was 28 (±23) years, in contrast to 24 (±24) years for patients infected with pathogenic *Y. enterocolitica*.

Several measures of disease severity (fever, need for antibiotics or hospitalization, occurrence of pseudo-appendicitis) did not differ between the groups. The occurrence of blood in stools was observed only in patients infected with pathogenic biogroups of *Y. enterocolitica*; however this was a rare symptom. No late complications (arthritis or erythema nodosum) could be ascertained by follow-up telephone call. One patient with a mycotic aneurysm of the aorta infected with *Y. enterocolitica* of biogroup 4 and septicaemia

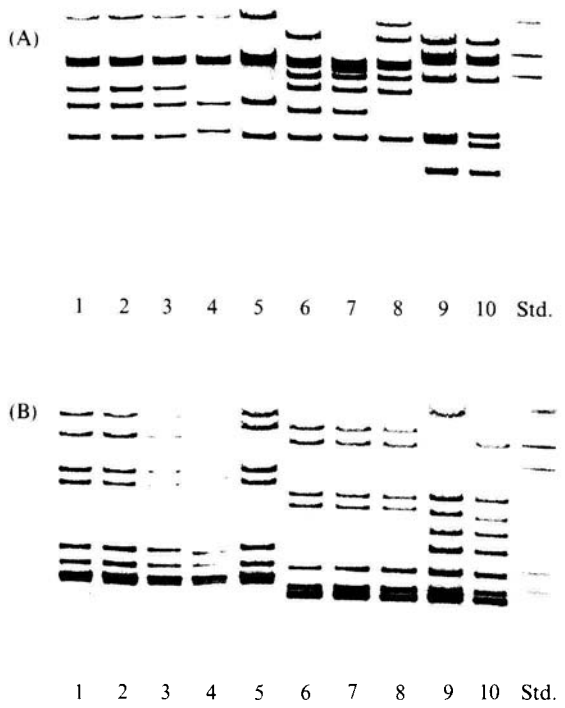

Fig. 1. *Pvu*II ribosomal RNA profiles of representative isolates of *Yersinia enterocolitica* biogroups. Panel A: profiles generated with the 16S ribosomal RNA probe. Panel B: profiles generated with the 23S ribosomal RNA probe. Lanes 1–3: human isolates belonging to pathogenic biogroup 4 (546–93, 1100–93, 638–93), lane 4: human isolate of pathogenic biogroup 2 (5865–92), lane 5: human isolate of pathogenic biogroup 3 (1775–92), lanes 6–8: representatives of common ribotypes among human biogroup 1A isolates (1139–93, 896–93, 132–93), lane 9: ribotype of the only human isolate of biogroup 1A resembling environmental strains (1322–93), lane 10: representative environmental isolate of biogroup 1A from lake water [33]. Std: molecular weight standard, digoxigenin-labelled lambda DNA restricted with *Hind*III.

died. Because of the differences in age distribution observed between the groups, and because age had been shown to influence clinical presentation of yersiniosis in previous studies, the data were also subjected to a stratified analysis. Neither the stratification of data by age, nor the exclusion of data from patients concomitantly infected with other enteric pathogens modified the observed lack of association between yersinia biogroups and symptoms in any way. Symptoms of pseudoappendicitis were not observed in children, and the youngest patient with this complication was 17 years of age. Ribotyping with *Pvu*II and 16S as well as 23S ribosomal RNA probes was performed for all the *Y. enterocolitica* strains included in the study in order to assess their epidemiology. Preliminary testing with different enzymes, including *Nar*I, *Sac*I, *Bss*HII, *Eco*RV, *Bam*HI, *Cla*I, *Nco*I, *Bcl*I, and *Kpn*I had shown *Pvu*II to give the most discriminatory profiles. The results for representative isolates are shown in Fig. 1. All isolates of biogroup 4 showed the same ribopattern, which was identical to the profiles of biogroup 3 strains. All strains belonging to biogroup 2 also had a conserved pattern. Bands of 4·4, 8·0 and > 10 kb were shared between strains of biogroups 2, 3 and 4. In contrast, strains of biogroup 1A had more varied patterns, even if two groups of profiles could be clearly recognized. All but one of the patient isolates of biogroup 1A belonged to the first group of profiles, represented by strains 1139–93, 896–3, and 132–93 in Fig. 1. An environmental isolate included for comparison in the rightmost lane of Fig. 1 belonged to a second group of profiles, predominant in the environment, and isolated from only one patient in the study.

DISCUSSION

The study was prompted by the observation in previous years that many strains of *Y. enterocolitica* isolated from faeces of patients with gastroenteritis had belonged to the biogroup 1A, traditionally regarded as being nonpathogenic. Almost 40% of human *Y. enterocolitica* strains received during the study period were of the biogroup 1A. The sample may have been somewhat biased, but non-pathogenic isolates are by no means rare, and the frequency of their occurrence observed here is consistent with reports by other investigators [9, 22, 23]. Three approaches could be considered to measure the potential clinical significance of biogroup 1A isolates of *Y. enterocolitica*. The first, and most straightforward, would be to show that such isolates are found more frequently among patients with gastroenteritis than among healthy controls. The strains used in the present study have necessitated culturing of about 13000 faecal samples in the participating laboratories, and obtaining an equivalent number of control specimens, mostly from children, is not considered feasible. Only one study has used this approach so far, and the authors reached a conclusion very similar to ours [9]. A second approach to establish the pathogenicity of biogroup 1A of *Y. enterocolitica* would be the use of an animal model. However, present mouse and rat models focus on systemic disease caused by yersiniae, i.e. septicaemic infections and post-infectious arthritis [24]. No simple animal models which entirely reproduce human intestinal disease due to *Y.*

enterocolitica have been described so far, even if the role of the *yst* enterotoxin has been studied in rabbits [25]. A third approach was therefore used in the present study. The clinical syndromes associated with *Y. enterocolitica* infections of strains belonging to pathogenic biogroups were compared with symptoms among patients, in whom a biogroup 1A of *Y. enterocolitica* had been isolated from a faecal sample. The reasoning was that the virulence attributes present in pathogenic strains but absent from the biogroup 1A isolates might affect the clinical course of illness. This would allow some estimation of the contribution of established virulence factors to the generation of symptoms in intestinal yersiniosis in humans.

For this comparison to be valid, genetic methods were used in addition to full biotyping in order to ascertain that the patient strains included in the comparison of clinical symptoms contained the full set of known virulence attributes specific for pathogenic *Y. enterocolitica* isolates. The correlation between biogroups and presence of *ail* as well as *yst* genes was excellent in this set of fresh clinical isolates, as expected on the basis of previous reports [26]. Interestingly, the only exceptional biogroup 1A isolate hybridizing with the *ail* probe was associated with symptoms of pseudoappendicitis in the patient from whom it had been isolated. However, instead of the expected 2·2 kb fragment, two *Ava*I fragments of 10 kb and > 23 kb hybridized with the *ail*-probe on Southern blots of this strain (results not shown). Ribotypes also strongly correlated with biogroups of *Y. enterocolitica*. The conserved profile of biogroup 4 reported here corresponds to the *Nci*I ribotype II found among European isolates described in a previous report [27], and a closely related ribosomal RNA profile was found among all pathogenic biogroups of yersiniae (Fig. 1).

No significant differences in clinical presentation were found between the patient groups infected with either pathogenic *Y. enterocolitica* biogroups or strains of the non-pathogenic biogroup 1A. In a previous report, significant differences in clinical symptoms associated with *Y. enterocolitica* biogroups had been found, but no information on virulence traits were given in that paper [22]. The chromosomal virulence genes *ail* and *yst*, as well as the virulence plasmid, which are found exclusively among *Y. enterocolitica* strains belonging to pathogenic biogroups, may fail to influence the clinical symptoms of intestinal infection, and may be of more importance in cases of disseminated disease. Some evidence has already been presented to show that the invasion-associated *inv* gene, universally present in all *Y. enterocolitica* isolates, may be critical in mediating intestinal disease [28]. Thus, it is not too surprising to find biogroup 1A isolates associated with disease, and all our isolates have been shown to hybridize to an *inv* probe (results not shown).

The gastrointestinal symptoms observed among the study patients tended to be generally milder than in other reports [29, 30]. These relatively mild symptoms may be explained by the fact that only 40% of patients were below the age of 10 years, as age has been shown to influence strongly the clinical presentation of disease due to *Y. enterocolitica* of biogroup 4 [31]. However, the subjects included in the present study represent the age range of patients likely to be encountered in a sample of yersiniosis cases from the general population [32]. The fact that all strains reported on here were isolated by direct plating of a faecal specimen further supports their clinical significance.

No definite proof of the pathogenicity of all biogroup 1A isolates can be based on the data presented. However, there is evidence that some biogroup 1A strains may be host-adapted and, perhaps, pathogenic. Molecular evidence for the occurrence of human-adapted biogroup 1A has been reported before. Clear differences between environmental and human isolates of biogroup 1A strains of *Y. enterocolitica* had been shown by multilocus enzyme electrophoresis [33]. The ribotyping results presented here support this grouping. Environmental *Y. enterocolitica* isolates from the study cited above [33] showed 16S ribotypes clearly different from the majority of patient strains (Fig. 1). Only one of the patient isolates belonging to biogroup 1A showed a profile resembling the environmental strains. The ribosomal RNA profiles of the remaining patient strains of biogroup 1A on the other hand, were strongly conserved. This group of strains with related multilocus enzyme electrophoresis patterns and similar ribotypes may represent a cluster of pathogenic biogroup 1A isolates of *Y. enterocolitica*.

The limited clinical differences observed between patients infected with biogroup 1A compared with those infected with pathogenic biogroup isolates of *Y. enterocolitica*, would suggest that clinical laboratories could minimize resources needed for biogrouping. The usefulness of different phenotypic tests for this purpose has already been evaluated by several investigators [26, 34]. Some points seem worth empha-

sizing. The discrimination between pathogenic and non-pathogenic biogroups by sodium acetate utilization has not been reported before. We also found the detection of calcium dependence and congo-red binding on CR-MOX medium [15] to be very reliable for recognition of pathogenic yersiniae. This was due to the fact that none of the strains analysed had lost their virulence plasmids. In fact, we have never observed plasmid loss in our collection of *Yersinia* strains, even after repeated passages *in vitro*, if care was taken not to use incubation temperatures above 30 °C. The poor specificity of slide agglutination for detection of pathogenic yersiniae was due to the fact that 27% of the non-pathogenic isolates reacted in the O5 serum. For serogroups O3 and O9, there was an excellent correlation between pathogenicity and slide agglutination. It is known that O5, 27 is a heterogeneous serogroup [35], comprising both pathogenic and non-pathogenic isolates. Therefore, except for serogroups O3 and O9, the local distribution of strains reacting in the O5 serum has to be ascertained with other methods before relying on slide agglutination for pathogenicity determination. However, none of the biochemical tests or sera mentioned above are easily available from commercial sources. Therefore, testing for aesculin hydrolysis on a commercial bile aesculin agar has been found to be the most convenient and inexpensive method for distinguishing pathogenic (aesculin negative) from non-pathogenic (aesculin positive) biogroups of *Y. enterocolitica*. Accumulating biogrouping data from more patients in different parts of the world may help to resolve some of the questions raised by this report.

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